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(DREAMS TM): Clinical and Basic Science Projects

PRINCIPAL INVESTIGATOR: Ward Casscells, M.D.

CONTRACTING ORGANIZATION: The University of Texas Health Science
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PROJECT TITLE: "Disaster Relief and Emergency Medical Services" (DREAMS) I.A.
THE UNIVERSITY OF TEXAS-HOUSTON HEALTH SCIENCE CENTER
PRINCIPAL INVESTIGATOR: Ward Casscells, M.D.
GRANT NO: DAMD17-98-1-8002
REPORTING PERIOD: 1/1/00 through 3/31/01

Investigator Name: James T. Willerson, M.D.

1. A. Administrative Section

We have used these funds to provide administrative support for DREAMS 2000. There is a need for secretarial assistance, coordination of the various research programs, statistical analysis assistance and funds for miscellaneous items required by investigators in this project.

All salaries are in accordance with the policies and procedures of the University of Texas-Houston Health Science Center. Fringe benefits are calculated at a rate of 25.6% for salaries up to \$40,000 and 20.6% for salaries over \$40,000.

Dr. Ward Casscells is the Principal Investigator for DREAMS and some financial support for his level of effort is requested in this section. Dr. James Willerson is an Associate Principal Investigator and financial support for his level of effort is also included in this request. Daniel Fu is the Administrator for the DREAMS program. He coordinates research schedules, administrative reports, purchasing activities, and serves to help each investigator solve administrative problems that arise. Funds are also requested for statistical assistance for data evaluation in each of the research sections and for office supplies (including paper, pencils, pens), storage items, computer costs, etc. for each of the investigators.

Final Yearly Progress Report: April 1, 2000- March 31, 2001

Project I.B.: Mechanisms of Cardiomyocyte Injury in Shock

Investigator Name: L. Maximilian Buja, M.D.

New Figures I-IV are on the final page of this report.

Introduction: The original work proposed was to investigate the pathway leading to cardiac myocyte cell death due to endotoxin (LPS). LPS is the major component from the outer bacterial cell wall that leads to profound and diverse effects in mammalian cells, contributing to the release of pro-inflammatory cytokines (interleukin -1 and TNF-alpha) and depression in cardiac function. Cardiac resistance to endotoxemic injury may occur in adult heart after exposure to a sublethal dose of LPS and this response requires an increase in de novo protein synthesis. During the course of our studies in the last year, we have uncovered an innate protective phenotype of the newborn heart against endotoxin-induced cell death. Together, these two observations in adult and neonatal heart suggest that the heart is able to mount an effective defense against endotoxin-induced cell death. The pathway by which this occurs is a characteristic of the developing heart and can be primed in the adult cardiac myocyte by de novo expression of protective genes.

For these experiments, we built on our past work demonstrating that neonatal cardiac myocytes were subject to programmed cell death when cultured in the presence of saturated fatty acids in high physiological molar ratios. Therefore, neonatal hearts can be induced to undergo programmed cell death (apoptosis) and we have dissected the pathway by which this occurs (1-3). In our studies with LPS, a known initiator of programmed cell death in the heart, we were surprised to learn that neonatal myocytes are resistant to the LPS cell death-signaling cascade (even though the same cells were capable of undergoing lipo-apoptosis). We first tested the hypothesis that reactive oxygen species (ROS) are more effectively detoxified by anti-oxidant pathways such as superoxide dismutase (SOD) in neonatal heart. High SOD activity would protect neonatal cardiac myocyte from programmed cell death by detoxification of increased LPS-induced, cytosolic superoxide production. The Zn/Cu ion chelator, diethyldithiocarbamate (DDC) is known to cause apoptosis in other cell types (4) by inhibition of cytosolic superoxide dismutase, increasing superoxide levels in the cells and activating the NFkB pathway by a redox mechanism. Using lucigenin as a fluorescent indicator, we were able to demonstrate that DDC indeed increased the superoxide levels in the cardiac myocyte. DDC augmented the NFkB gel shift (using both electrophoretic mobility shift assays as well as confocal microscopy) elicited by exposure of the cells to endotoxin or palmitate (**Progress Report I, April -June, 2000: containing Figures 1-4**). In response to inhibition of SOD and increased nuclear translocation of NFkB, there were small (20-60%) increases in caspase-3 like activities compared to myocytes in the absence of LPS. In contrast, the 4-fold increase in superoxide in the DDC-treated myocytes (measured by lucinigen luminescence) suggests that neonatal cardiac myocytes possess innate protection from the redox-mediated pathway of programmed cell death. The effect of LPS and DDC on NFkB nuclear translocation with the potential of binding to DNA enhancer elements indicated to us that in the neonatal cardiac myocyte, NFkB is increasing transcription of "survival" genes or that survival mediated pathways are "turned on" in direct response to LPS signaling.

Our attention turned to protective pathways that are mediated by the substrates of PI3K, i.e. Akt/ protein kinase B. This pathway has been shown to be activated by TNF-alpha (5) and to protect the adult cardiac myocyte against ischemia-mediated cardiac apoptosis (6). It has been suggested that the downstream substrate of this pathway is the pro-apoptotic gene, BAD, which is inactivated by phosphorylation, and the upregulation of the transcriptional activator CREB that activates the anti-apoptotic gene, Bcl-2. Consistent with

this expectation, we discovered that addition of LPS to neonatal cardiac myocytes increased Akt phosphorylation by 2 hours. The increased phosphorylated ratio of P-Akt/ Akt, maintained a plateau for as long as 20 hr (**Figure 6 contained in Progress Report I, and Figure 2 contained in the Proposal Renewal Application**). The phosphorylation of Akt by PI3K can be mediated by the inflammatory cytokine, TNF-alpha (5). Addition of LPS to the neonatal cardiac myocyte culture produces an 80-fold increase in TNF-alpha secretion at one hour, with subsequent exponential increases thereafter (**Figure I**). Temporally, the secretion of TNF-alpha, interaction with its receptor and activation of the PI3K pathway are consistent with either: (1) increased transcriptional activation as a result of NFkB translocation (**Figure 1 contained in the Proposal Renewal Application**) and accompanying IkB-alpha degradation (**Figure 1 contained in the Progress Report, January 15, 2001**), or (2) activation of the PI-3K/ Akt pathway (see above). To test the role of the PI3K survival pathway on the protection of LPS-treated myocytes from apoptosis, we exposed the cardiac myocytes to the PI3K inhibitors LY294002 and Wortmannin. Pretreatment with either inhibitor blocked the phosphorylation of Akt (**Figures 3 and 4 contained in the Progress Report, October 15, 2000**). However, there was no effect on either caspase 3 activation (and therefore progress to cell death) or NFkB nuclear translocation which remained high (**Figures 5 and 6 contained in Progress Report, October 15, 2000**). The data suggest that the inhibition of the PI3K survival pathway is not by itself a mediator of the resistance of the neonatal myocyte to endotoxin. Another potential pathway that mediates anti-apoptotic sequellae is the Erk/Ras pathway. We have demonstrated that Erk 1 /2 is phosphorylated in response to LPS as early as 30 min following LPS exposure (**Figure 1 contained in Progress Report, October 15, 2000**). These results open the possibility that the Erk/Ras pathway may be activated by the non-receptor tyrosine kinase, Src.

A second avenue of protection has recently been explored and relates to the "pre-conditioning" effect of cyclo-oxygenase -2 (COX-2) induction (7) by preincubation of adult myocytes with LPS. We found significant quantities of COX-2 enzyme in neonatal myocytes and in adult myocytes using immunoblot analysis (**Figure II**). This protein was slightly increased in amount following LPS exposure in adult myocytes. Increases in COX-2 protein in adult myocytes coincides with the findings of induction of COX-2 activity by LPS preconditioning. In the neonatal myocyte, however, inhibition of COX-2 by NS398 had no effect on caspase-3 activation (**Figure 4 contained in the Progress Report, January 15, 2000**). The early effects of COX-2 activation involve the production of the pro-inflammatory prostaglandins, PGE, whereas late effects of COX-2 activation involve the production of bioreactive anti-inflammatory cyclopentenones. The anti-inflammatory CydPGJ₂ is formed from PGD₂ and interferes with NFkB transcriptional activity. We have demonstrated that CydPGJ₂ prevents nuclear translocation of NFkB and phosphorylation of IkB alpha (**Figure III**), inhibits secretion of TNF-alpha when added prior to LPS (**Figure I: middle panel vs last panel**) and results in a 10-fold activation of caspase 3-like activity (**Figure IV**). We find these results to be extremely exciting as they suggest (in agreement with other investigators) that gene transcription by NFkB is cardioprotective and that synthesis of the anti-inflammatory PGJ₂ may be suppressed in the neonatal myocytes compared to the adult. We have one abstract accepted for presentation on these late-breaking data at the World Congress of the International Society for Hear Research in Winnipeg Canada, July 6-11, 2001 (see below).

References:

1. Hickson-Bick, D.L., Buja, L.M. and McMillin, J.B. (2000) J. Mol. Cell. Cardiol. 32: 511-519.
2. (2000) Am.J.Physiol.Heart Circ.Physiol. 279: H2124-H2132.

3. Sparagna, G.C., Hickson-Bick, D.L., Buja, L.M. and McMillin, J.B. (2001) Antioxidants and Redox Signaling.
4. Siwik, D.A., Tzortzis, J.D., Pimental, D.R., Chang, D.L., Pagano, P.J., Singh, K., Sawyer, D.B. and Colucci, W.S. (1999) Circ. Res. 85: 147-153.
5. Sparagna, G.C., Hickson-Bick, Buja, L.M. and McMillin, J.B. Ozes, O.N., Mayo, L.D., Gustin, J.A., Pfeffer, S.R., Pfeffer, L.M. and Donner, D.B. (1999) Nature 401: 82-85.
6. Kurrelmeyer, K.M., Michael, L.H., Baumgarten, G., Taffet, G.E., Peschon, J.J., Sivasubramanian, N. Entman, M.L. and Mann, D.L. (2000) Proc. Natl. Aca. Sci. U.S.A. 97:5456-5461.
7. Shinmura, K., Tang, X.-L., Wang, Y., Xuan, Y-T., Liu, S-Q., Takano, H., Bhatnager, A. and Bolli, R.(2000) Proc. Natl. Acad. Sci. 97: 10197-10202.

Published Abstract:

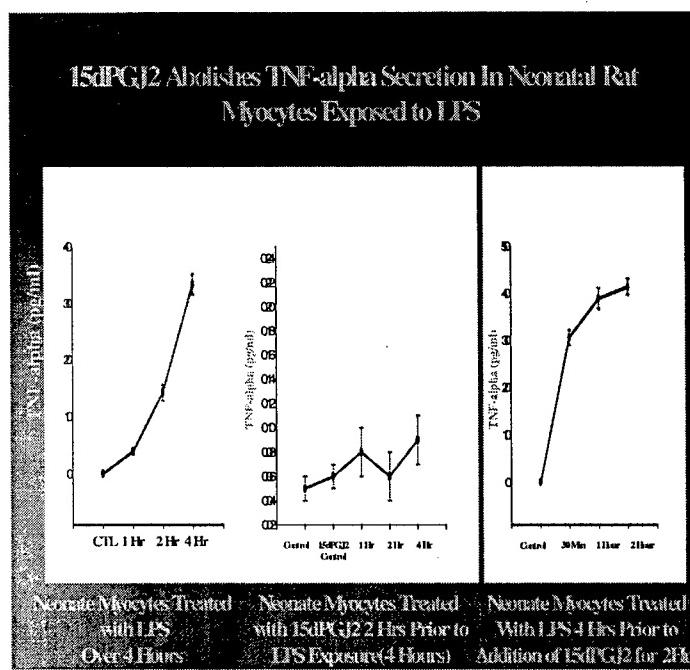
CYCLOPENTENONE MEDIATES APOPTOSIS BY LPS IN NEONATAL CARDIOMYOCYTES

Chad Jones, Jeanie B. McMillin & L. Max Buja. Dept. Pathology, University of Texas Medical School, Houston, TX, USA.

Neonatal cardiac myocytes are resistant to LPS-induced apoptosis despite strong activation of NF κ B and secretion of TNF- α . Phosphorylation of the cytoprotective proteins, Erk-1 and -2 and Akt occurs within 30 min of LPS and remains high for 3 hours. This time course is parallel with NF κ B translocation into the nucleus and I κ B- α degradation as demonstrated by EMSA and immunoblotting. Inhibition of PI3K by wortmannin or LY294002 does not induce cell death (measured by no change in caspase-3 like activity) or alter NF κ B translocation. We find that COX-2 protein is highly expressed in neonatal cardiomyocytes. COX 2 expression has a diverse array of biological responses, including cardiac preconditioning. COX 2 synthesizes both pro-inflammatory PG and the anti-inflammatory cytokine, cyclopentenone (CyPG), which inhibits IKK- β . Addition of CyPG following 0.5 to 1 hr in LPS produces a 9-fold increase in caspase 3 and abolishes nuclear translocation of NF κ B in a dose-dependent manner. We conclude that neonatal myocytes lack the pathway for CyPG production and that activation of NF κ B switches on a multitude of cytoprotective mechanisms to promote myocyte survival (DAMD#17-98-1-8002 to LMB & JBM).

Figures I-IV on the following page.

Figure I
Figure II



Cyclo-oxygenase 2 Synthesis Increases 40% in Cultured Adult Rat Cardiac Myocytes Exposed to 1ug/ml LPS

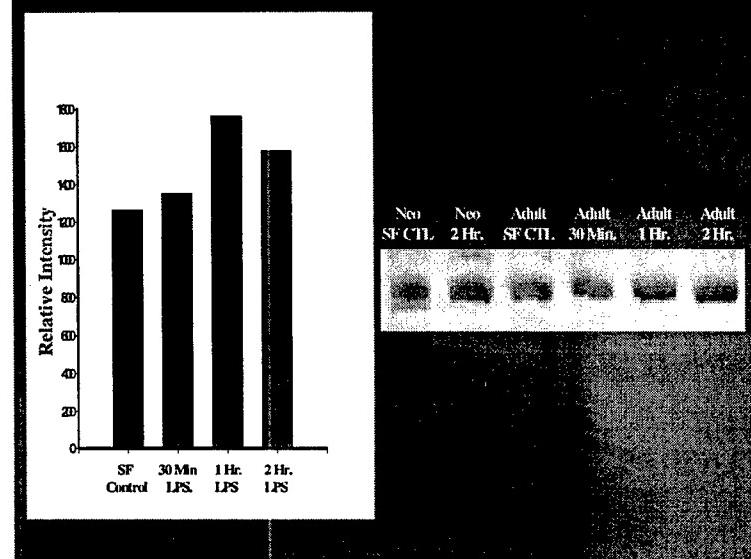
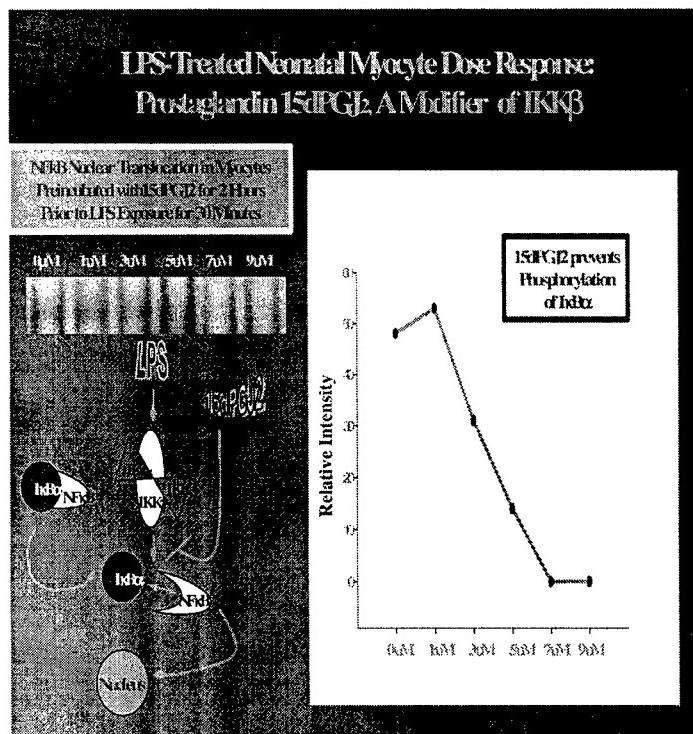
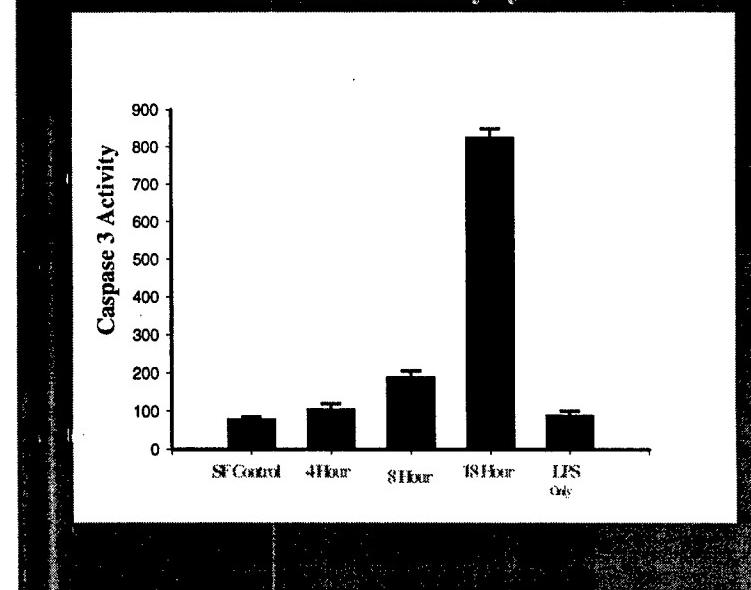


Figure III
Figure IV



Incubation with LPS 1 Hour Prior to Adding the Cyclopentenone Prostaglandin 15dPGJ2 Leads to Massive Caspase 3 Activation in Neonatal Cardiac Myocytes



PROJECT TITLE: "Disaster Relief and Emergency Medical Services Project
(DREAMS TM): Clinical and Basic Science Projects"
THE UNIVERSITY OF TEXAS - HOUSTON HEALTH SCIENCE CENTER
PRINCIPAL INVESTIGATOR: Ward Casscells, M.D.
GRANT NO: DAMD17-98-1-8002
REPORTING PERIOD: 4/1/00 - 3/31/01

Project #/Title:I.C.1 Apoptosis in wound healing

Investigator Name: Yong-Jian Geng

Investigator Signature: _____

Progress Report: Describe scientific progress for the quarter in terms of the tasks or objectives listed in the statement of work for this project. Explain deviations where this is not possible. Include data where possible. Present a brief statement of plans or milestones for the next quarter. Do not exceed two pages.

The objectives of the proposed research are to determine molecular mechanisms underlying apoptosis of inflammatory cells and vascular cells during wound healing. We have obtained experimental data that can be divided into three parts:

1. Analysis of cholesterol crystallization in macrophages. One of hallmarks for atherosclerosis is the formation of cholesterol crystals. Accumulation of the crystals in the arterial wall with atherosclerosis can cause atheroembolism and acute vascular syndrome. Occlusion of the arteries by cholesterol crystals often induces irreversible ischemic damage to the tissue. We examined cholesterol crystals and apoptosis in macrophages ($M\phi$) in human carotid arteries and apolipoprotein-E deficient (apoE-null) mice, and in phorbol ester-stimulated human THP-1 monocytic cells exposed to different temperatures. DNA labeling and immunohistochemistry revealed that both human carotid and apoE-null aortic plaques contained numerous apoptotic macrophages surrounded by cholesterol crystals. In the "hot" regions rich in inflammatory components such as T cells and monocytes, the crystals were less abundant. Treatment of cultured THP-1 macrophages with 7-ketcholesterol (20 μ g/ml), an oxysterol component of oxidized lipoproteins, but not the same amounts of free cholesterol and 25-OH-cholesterol at 37°C for 48 hours, promoted sterol crystal formation. Cell viability assay, *in situ* detection of DNA fragments, and agarose gel electrophoresis revealed increased apoptosis in the 7-ketcholesterol-treated $M\phi$ (29.6% \pm 6% vs. 5.8% \pm 1.4% in controls). Fluorescence microscopy showed the presence of the crystals inside and on the surface of lipid-laden foam cells. Fewer crystals were found in the cells undergoing apoptosis. X-ray diffraction confirmed the presence of 7-ketcholesterol crystalline domains (d space 35.8 Å) in the membrane of $M\phi$ incubated with 7-ketcholesterol. When cultured at 40°C for 48 hours, the crystal formation markedly declined by 82% in $M\phi$, while apoptosis increased by 85% in $M\phi$. In the absence of the oxysterol, increasing temperature neither affected the cell viability nor changed the membrane crystalline domains. These results suggest that hyperthermia enhances apoptotic effect of the oxysterol, but reduces cholesterol or oxysterol crystal formation, which may in turn influence the plaque stability. We will continue this study by analyzing the chemical components of the cholesterol crystals and determining the effects of lipid-binding proteins on the formation of cholesterol crystals and apoptosis. We will examine the role for scavenger receptor in regulation of apoptosis.

2. Determination of apoptosis gene expression by microarray technology. We have examined the genes coding for proteins that participate in regulation of apoptosis, DNA synthesis, repair and recombination in Apolipoprotein-E deficient mouse aortas by cDNA arrays and immunohistochemistry. cDNA arrays revealed expression of several known apoptosis-regulating genes including those coding for caspases and the members of Bcl-2 protein family in both apoE-null and wild type mice. In apoE-null mice, expression of the genes coding for TRAIL, Bax, RIP and TDAG51 appeared to be attenuated, while expression of the genes for clusterin, Fas, DAD-1, and FAF1 were increased, when compared to the wild type controls. Interestingly, we observed higher levels of expression of several genes involved in DNA repair, synthesis, and recombination. Among them were HR21spA, nucleoside diphosphate kinase B, and glutathione S-transferase. However, PMS2 DNA mismatch repair protein and translin, a recombination binding protein, were found at lower levels in apoE-null mice than that in wild type mice. We plan to selectively analyze clusterin expression and function in the next year studies if this project is granted.

3. CD1 protein expression in smooth muscle cells and inflammatory cells. We have recently crossed CD1-knockout mice with those lacking Apolipoprotein-E. Genotyping and gross evaluation revealed that CD1/apoE double knockouts survive without major defects in embryonic and post-birth development. In this study, we examined CD1 in the cells previously isolated from these animals. Further experimental designation is aimed at investigating the difference in response to injury between the heterozygous and homozygous knockouts and wild type mice. We will also analyze the phenotypical alterations and functions of T cells, macrophages and vascular smooth muscle cells. In the study of apoptosis, we will examine expression of the death regulating genes, Fas, Fas ligand, caspases and members of the Bcl2 gene family.

Publications:

1. **Geng** YJ. Biologic effect and molecular regulation of vascular apoptosis in atherosclerosis. *Curr Atheroscler Rep.* 2001, 3(3):234-42.
2. Liao HS, Kodam a T, **Geng** J. Expression of class A scavenger receptor inhibits apoptosis of macrophages triggered by oxidized low density lipoprotein and oxysterol. *Arterioscler Thromb Vasc Biol.* 2000, 20(8):1968-75.
3. Asai K, Kudej RK, Shen YT, Yang GP, Takagi G, Kudej AB, **Geng** YJ, Sato N, Nazareno JB, Vatner DE, Natividad F, Bishop SP, Vatner SF. Peripheral vascular endothelial dysfunction and apoptosis in old monkeys. *Arterioscler Thromb Vasc Biol.* 2000, 20(6):1493-9.
- 4: Vatner DE, Yang GP, Geng YJ, Asai K, Yun JS, Wagner TE, Ishikawa Y, Bishop SP, Homcy CJ, Vatner SF. Determinants of the cardiomyopathic phenotype in chimeric mice overexpressing cardiac Gsalpha. *Circ Res.* 2000, 86(7):802-6.

R O U G H D R A F T

**DREAMS ANNUAL REPORT
I.C.2**

Hot Plaque. (Exact title and project number to be supplied later.) (Dr. C - Mort said the title was "Thermal Detection of Atherosclerotic Plaque" but Rosie's Dreams Project list shows " Infrared Spectroscopic Diagnosis of Vulnerable Atherosclerotic Plaques" (Project # shows I.C.2) - PLEASE ADVISE.

This project continues to proceed on schedule and to generate successful spinoff projects. Because of the central role of inflammation in so many diseases, including infection, wound healing, autoimmune diseases, cancer, heat and atherosclerosis, we have focused on the role of inflammation and the progression of atherosclerotic plaque because, as the ultimate event triggering myocardial infarction and stroke, it remains the leading cause of death in the United States. Moreover, the World Health Organization estimates that myocardial infarction and stroke will be the leading causes of death worldwide by 2010. Plaque rupture accounts for some 60 percent of myocardial infarctions and plaque erosion for the remaining 40 percent. Strokes due to carotid thrombosis are similar except that a debatable percentage of carotid occlusions are caused by plaque dissection.

Inflammation is the common denominator that links both plaque rupture and plaque erosion. Heretofore, there has been no method of detecting these foci of inflammation. Thus, despite the many advances in cardiology, it remains true today that 40 to 50 percent of heart attacks occur in people with no prior symptoms. Seventy percent of heart attacks are caused by the rupture or erosion of a plaque which, at the most recent angiogram, conferred less than a 70-percent-diameter stenosis. Since these plaques are, by definition, not detectable by stress testing, and since angiography cannot accurately detect inflammation, cardiologists are still unable to predict and prevent the majority of myocardial infarctions.

We found several years ago that living plaques exhibit thermal heterogeneity. The warmer areas correlated with cell density. The acellular areas were cool. As shown in the attached manuscript by B. Malik, M.D., et al, we now report that the heat is most closely related to the density of macrophages.

Smooth-muscle cell density is unrelated to plaque heat. Further, we found that the correlation of heat to cell density is highest when the plaques are measured in a physiologic environment (37° C., i.e., body temperature) rather than in a living-but-cool state (i.e., a 24° C. room). Dr. Malik further showed that the heat is unrelated to the presence of Chlamydia pneumoniae, a candidate pathogen. Heat was modestly related to the serum cholesterol, a known stimulant to inflammation. Further evidence of the rule of inflammation is the fact that heat was inversely correlated with aspirin therapy, albeit modestly. Moreover, when plaques were removed and bathed *in vitro* in indomethacin, plaque temperatures cooled and became more homogeneous compared to tissue culture medium without indomethacin.

M. Madjid, also in our group, demonstrated that patients' oral morning temperatures fall approximately ten days after starting cholesterol-lowering statin medication such as simvastatin, atorvastatin or pravastatin (see attached). R. John et al (John R, Naguib S, Siadaty S, Naghavi M, Willerson JT, Casscells W. Correlation of Temperature, pH and Cell Density in Living Human Atherosclerotic Plaques. *European Heart J* 2000; 21(Abstr Suppl):250) and M. Madjid et al (Madjid M, Asif M, Naguib S, Siadaty S, Willerson JT, Casscells W, Naghavi M. Hot and Acidic Atherosclerotic Plaques are More Vulnerable to Rupture. *J Am Coll Cardiol* 2001; 37(Suppl A):1A-648A) went on to show that these inflamed areas are not only warm but acidic. These areas also tended to be yellow in appearance, whereas whitish calcified regions were associated with lower temperature and higher pH, presumably reflecting the fact that the areas of calcium salts are essentially acellular. Madjid et al further showed that the pH could not only be measured with a pH needle electrode but also could be imaged with pH-sensitive fluorescent dyes, confirming the electrode measurements.

B. The Role of Plaque Heat.

We are exploring the hypothesis that plaque heat is not just a byproduct of metabolic activity, to be discharged from the body by convection to the skin, radiation, perspiration, breathing, urination, etc. We hypothesized that plaque heat has numerous physical, chemical and biological effects, including the softening of plaque lipids, activation and inactivation of enzymes depending on their temperature optima, and gene expression. To this end, we

have designed a chip to measure the expression of an array of genes thought to be involved in atherosclerosis. We began with studies conducted by Y. Geng, M.D., Ph.D., in our group. Using APO-E-deficient knockout mice which spontaneously develop hypercholesterolemia and a type of atherosclerosis, Dr. Geng found that atherosclerosis was accompanied by the activation of genes involved in inflammation, cell division, lipid metabolism, apoptosis, oxidation and DNA repair. Dr. S. Anwar, in our group, has designed a chip which is now being made to further study these changes using reverse transcribed messenger RNA from human carotid specimens (See attached papers by Y. Geng and the attached report by S. Anwar.) (DR. CASSCELLS-MORT SAID THAT DR. ANWAR'S NAME SHOULD BE REPLACED WITH HIS NAME AND THAT THE GENE ARRAY TECHNOLOGY IS BEING DEVELOPED IN COLLABORATION WITH AFFYMETRICS AND ERKIN-PELMER INC. AND THERE IS NO REPORT - PLEASE ADVISE).

As another approach to examining the effects of heat, we experimented with living rabbit, mouse and freshly extracted human carotid atherosclerotic plaques and gently heated these plaques to the upper end of the fever range in organ cultures (tissue culture medium in a 37° incubator). In the previous DREAMS annual report, we described a down regulation of pro-inflammatory genes by heating at the upper end of the fever range (e.g. 40 to 41° C.) for 15 minutes: TNF alpha and its receptor, IL-1, MCP-1, etc. We next found that when we heated to the range of 41 to 43° C., this produced apoptosis, almost exclusively in the macrophage population. In the current reporting, K.Gul et al (Gul K, Naghavi M, Siadaty S, Casscells W, Cohen A, Willerson JT. *In Vivo Physiologic Heating of Rabbit Atherosclerotic Plaques Induces Macrophage Apoptosis: A New Approach to Plaque Stabilization.* *J Am Coll Cardiol* 2000; 35(Suppl A):244) examined whether this effect could be obtained *in vivo*. In 11 rabbits heated, using a cooled ablation catheter, to 42° C. for 15 minutes, the percentage of apoptosis in the intimal layer increased from 5.7 . 8.9 to 30.3 . 23.1. Most of these cells were macrophages, as shown by immunostaining. Tewatia et al (Tewatia T, Wu C, Xu X, Kil K, Willerson JT, Casscells SW, Gene Y-J. *Selective Induction of Apoptosis in the Atherosclerotic Aortas of Apolipoprotein-E Deficient Mice by Short-Thermal Therapy.* *J Am Coll Cardiol* 2001; 37(Suppl A):1A-648A) found that heating living aortas freshly removed from APO-E or wild-type mice at 42° in DMEM medium (vs. 37°) caused a 35 to 40 percent increase in the number of apoptotic nuclei, as judged by TUNEL *in situ* DNA end-labeling. Almost all of these cells were

located in the media of the APO-E-deficient mice. The apoptosis was verified by extraction of genomic DNA and examination of the electrophoretic laddering pattern characteristic of inter nucleosomal DNA fragmentation. The occurrence of apoptosis was further confirmed by electron microscopy and by immunohistochemistry with antibodies directed against caspase-9.

The above papers suggest the attractive possibility of using brief localized heating--delivered by heating elements, convection, infrared radiation, radiofrequency or ultrasound--to cause a transient down-regulation of inflammatory gene expression and, with slightly more heat, to selectively cause apoptosis of the macrophages. Since the macrophages are the cells which digest the plaque cap, causing rupture or erosion, this would likely be a beneficial effect. The durability of such an effect has yet to be studied, but work done by Willerson and colleagues (see the DREAMS project by E. Yeh, JT Willerson et al) suggests that macrophages develop in plaques over weeks. If the majority could be eliminated with a single 15-minute heating period, this would □buy time□ for other anti-inflammatory medications, cholesterol-lowering medications, antithrombotic medications, antioxidants, dietary changes, etc. to further stabilize the plaque.

A second potential application is that of restenosis, which still causes an appreciable morbidity cost and even mortality after percutaneous interventions on the coronary, carotid and peripheral arteries. Because inflammation is a predictor of which lesions will develop restenosis after intervention, B. Lal et al (Lal B, Guo B, Naghavi M, Willerson JT, Casscells W. Noninvasive Ultrasound-Induced Heating of Stents: Importance of Stent Composition. *J Am Coll Cardiol* 2000; 35(Suppl A)25) Isabel Alban-Rojas et al tested the response of various stent materials to ultrasound heating. Ultrasound heating was chosen because intravascular ultrasound is a procedure in current clinical use as a diagnostic in the assessment of plaques in pre- and post-intervention. Using a muscle phantom in which various stent-shaped materials were placed and monitoring the heating with multiple needle thermocouples, Lal et al used levels of therapeutic ultrasound approved by the FDA for treatment of skeletal muscle injuries (0.5-2.5 watts/cm.sq., 1-3 MHZ in continuous and pulsed modes). Some materials, such as nylon and some types of PVC, heated faster (1.5-15 times and more to 35° C. increment) than the surrounding muscle, whereas other materials did not heat preferentially. These included PTFE,

latex, Teflon, ceramic, Delran and Lexan. Steel stents heated modestly (2° C. over 15 minutes).

Naguib et al (Naguib S, Alban-Rojas I, Rojas G, Raffoul G, Siadaty S, Casscells SW, Naghavi M. Stent Diathermy Using Focused Ultrasound and Microwave. *European Heart J* 2000;21(ABstr Suppl):281) confirmed and extended the results of Lal et al, using Palmaz-Schatz stents embedded in a phantom monitored with thermocouples and fiberoptic sensors. Therapeutic ultrasound and microwave (2.45 GHz at 5.37 or 10.22 watts) heated the stent materials. Silicone heated more than polyurethane or PTFE in both ultrasound and microwave experiments.

Mohammadian et al (Mohammadian Y, Bailey M, Vaezy S, Ollos R, Madjid M, Lal B, Willerson JT, Naghavi M, Casscells SW. Stent Diathermy: Noninvasive Gentle Heating of Stent Using Focused Ultrasound as a Potential Treatment of End-Stent Restenosis. (Presented at the Cardiovascular Radiation Therapy V Symposium - Radiation Therapy V Syllabus, Abstract 51, pg. 69, 2/5/01) are exploring another approach, that of focused therapeutic ultrasound, which can in theory be used outside the body, replacing the need for intravascular ultrasound thermal therapy. As a first approach, they used a living bovine liver tissue phantom and a 2 MHZ extracorporeal focused ultrasound transducer. Heating was monitored using an infrared camera and by implanted thermocouples. Changes in temperature were also correlated with changes in ultrasound speed shifts. They found that temperature in the stented vessel phantom could be localized to the vessel wall, and, even in the presence of fluid flow, could be raised from 37° C to 42° C and maintained for 20 minutes. Moreover, they were able to monitor the temperature shift by its impact on the ultrasound image. These results were subsequently confirmed by S. Sanati et al (Sanati S, Vaezy S, Bailey M, Madjid M, Casscells W, Willerson J, Naghavi M. Localized Controlled Heating of Implanted Stent by Noninvasive Focused Ultrasound. *Am J Cardiol* 2000; 86(Suppl 8A):105i.

D. Detection of Hot Plaques.

The detection of hot plaques is desirable both to detect the plaques at risk and for monitoring of temperature in the event that thermal therapy indeed proves itself as a viable initial approach to plaque stabilization. To this

end, Gul et al (Gul K, O'Brien T, Siadaty S, Madjid M, Mohammadi RM, Willerson JT, Casscells W, Naghavi M. Coronary Thermal Sensor Basket Catheter: A Low-Cost Tool for Thermal Detection of Atherosclerotic Plaques. *Am J Cardiol* 2000; 86(Suppl 8A):23i) designed a 4 French catheter with nine built-in thermosensors placed on an expandable and externally controllable basket. The computer board contains specialized digital transistors for high-speed sampling. They also developed a custom software for real-time data acquisition tracking and thermographic imaging. They tested this in a circulating microbath. The thermocouples achieved a thermal resolution in the phantom blood flow model of 0.01° C. Further testing was conducted in six Watanabe hypercholesterolemic rabbits and five inbred, cholesterol-fed atherosclerotic dogs. Temperature heterogeneity was detected in the aorta of each animal.

Guo and colleagues (Guo B, Willerson JT, Bearman G, McNatt J, Malik B, Gul K, Casscells W. Design of an Infrared Fiber Catheter for Thermal Imaging of Atherosclerotic Plaques. *J Am Coll Cardiol* 2000; 35(Suppl A):37) are attempting to develop an alternative catheter based on infrared thermal imaging. In theory, such a catheter will give a more complete picture of the thermal heterogeneity of the vessel wall with higher spatial resolution and no need for direct contact with the vessel wall. The same system could likewise be used for non-contact and highly localized infrared heating. The first prototype consisted of a bundle of 100 micron diameter As₂S₃ chalcogenide glass fibers which transmit infrared radiation from 0.7 to 7 microns. This catheter was connected to a high-sensitivity indium antimonide infrared focal plane (?????) array detector. With this system, it was possible to obtain images with spatial resolution of 100 microns and thermal resolution of at better than 0.1° C. in living aortic tissues immediately after excision from Watanabe rabbits.

An alternative design was tested by Naghavi and colleagues (Naghavi M, Melling P, Gul K, Madjid M, Willerson JT, Casscells W, Asif M. First Prototype of a 4 French 180-Degree Side-Viewing Infrared Fiberoptic Catheter for Thermal Imaging of Atherosclerotic Plaque. *J Am Coll Cardiol* 2001; 37(Suppl A):1A-648A) developed a smaller (4 French catheter) for use in the coronaries. Nineteen chalcogenide fibers were arrayed so as to give a 180-degree fish-eye view of the vessel wall. Software algorithms were developed to provide real-

time image reconstruction of a 1-mm window with temperature color coding and similar results in the phantom to those reported by Guo et al.

In summary, over the past years this DREAMS project has added considerably to our understanding of the magnitude and causes and implications of temperature heterogeneity in plaque. Much work remains to be done in each of these areas, but clinical applications are already apparent and, as they have been confirmed by several other research groups in the past year, they have led to the development of efforts among several of the catheter companies to develop catheters to detect and even image vulnerable plaques by the heat that they emit and perhaps to treat them by heating them further to induce an anti-inflammatory effect. The DREAMS investigators are proud that one of these catheter companies, Volcano Therapeutics, Inc., has licensed patents owned by the University of Texas Health Science Center at Houston and the Texas Heart Institute. One of these patents was filed prior to the initiation of the DREAMS program, but another was filed using data supported by the DREAMS program and is therefore available for royalty-free use by the United States Army. For further review of this rapidly growing field, please see the attached review, □New Developments in the Detection of Vulnerable Plaque,□ by Naghavi et al. The DREAMS investigators wish to call the Army's attention in particular to two studies that have grown out of the hot plaque project. Naghavi et al (Naghavi M, Barlas Z, Siadaty S, Naguib S, Madjid M, Casscells W. Association of Influenza Vaccination and Reduced Risk of Recurrent Myocardial Infarction. *Circulation* 2000; 102:3039-3045) found in a case-controlled study of 218 patients with chronic coronary disease followed at Memorial Hermann Hospital and The University of Texas that those who received influenza vaccination had a 67 percent reduction in their risk of myocardial infarction over the next six months or so. This paper attracted considerable attention, and influenza vaccination is now reported to reduce the risk of sudden cardiac death and stroke also, but all of these studies have been case-control studies rather than randomized controlled trials, which are more definitive. We are in the process of trying to access some of these databases or start such a randomized controlled trial. In the meantime, we have tried to model this effect in hypercholesterolemic APO-E deficient mice. As shown in the attached manuscript, □Influenza Infection Causes Acute Subendothelial Infiltration of Macrophages and CD3-Positive Cells and Smooth-Muscle Cells with Superficial Platelet Aggregation in Aortic Atherosclerotic Plaque of APO-

E Deficient Mice,□ which has now been accepted for publication in *Circulation*, we found a striking effect of exposing APO-E mice to influenza A (the same strain to which humans are susceptible). Almost all of the animals showed an exacerbation of their atherosclerosis, in most cases severe enough to cause functional stenosis. No such finding has ever been reported previously in the mouse model. Moreover, there were instances of coronary lesions and there was at least one documented instance of plaque erosion. The plaques were highly inflamed and often covered with a platelet or platelet fibrin deposit. This is the first study to show that influenza infection acutely exacerbates atherosclerosis. Moreover, as RT-PCR showed no virus in the plaques itself, it strongly suggests that the effect is primarily an immune reaction and/or an effect on coagulability. These findings may have implications for public health programs--which to date have not been successful in persuading all of those at risk to receive the vaccine. These findings may also lead to a new model by which mechanisms and therapies for acute coronary syndromes can be studied efficiently. Current and future studies are described in the recent application for DREAMS renewal for fiscal years 2001-2002 (submitted June 2001).

Appendix:

Effect of Atorvastatin (Lipitor®) Therapy on Morning Temperature in Patients With Cardiovascular Disease

Background:

There is increasing evidence that treatment of patients with cardiovascular disease with HMG-CoA reductase inhibitors reduce their risk of major cardiac events, coronary procedures, CHD mortality, and overall mortality. Moreover, non-lipid-lowering beneficial effects of statins are now recognized. The recent release of the Executive Summary of the Third Report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III) has broadened indications for them.

Recognizing that atherosclerosis is often diffuse and inflammatory, and considering the need for monitoring the efficacy of statins (as promoted by all treatment guidelines), we have sought to investigate whether body temperature is lowered by the use of statin drugs (reflecting reduced inflammation) and if it can be used to monitor the effect of statins. Body temperature modulations, if proved to be true, can also be used to shed light on other metabolic effects of statins.

Method and Results:

We have investigated a population of hyperlipidemic patients with a history of cardiovascular disease who were not already receiving statins and were recruited from an outpatient clinic. Those with factors affecting body T (e.g. hyperthyroidism, hypothyroidism, active inflammatory or infectious disease, etc.) were excluded from the study. Various statins in varying doses were prescribed by their physicians. Patients were instructed to record their oral temperature for three minutes on awakening (before arising). Mean T for two days before the initiation of therapy was used as the baseline. We studied 31 patients (24 men and 7 women) aged 57 ± 10 years. All patients recorded their T for at least 19 days (19-37 days, median= 22 days, SD=4 days). Mean T before



REPLY TO
ATTENTION OF

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MD 21702-5012

MCMR-RMI-S (70-1y)

15 May 03

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for this Command. Request the limited distribution statement for the enclosed accession numbers be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.
2. Point of contact for this request is Ms. Kristin Morrow at DSN 343-7327 or by e-mail at Kristin.Morrow@det.amedd.army.mil.

FOR THE COMMANDER:

Encl

Phyllis Rinehart
PHYLLIS M. RINEHART
Deputy Chief of Staff for
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